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Phylogeographic Subspecies Recognition in Leopards (*Panthera pardus*): Molecular Genetic Variation

SRIYANIE MITHTHAPALA,*†§ JOHN SEIDENSTICKER,† AND STEPHEN J. O'BRIEN‡

*Biological Carcinogenesis and Development Program, Program Resources Incorporated, National Cancer Institute—Frederick Cancer Research and Development Center, Frederick, MD 21702-1201, U.S.A.
†National Zoological Park, Smithsonian Institution, Washington, DC 20008, U.S.A.
‡National Institutes of Health, Laboratory of Viral Carcinogenesis, National Cancer Institute—Frederick Cancer Research and Development Center, Frederick, MD 21702-1202, U.S.A.

Abstract: *The incorporation of precise definitions for taxonomic units into wildlife legislation has necessitated the reevaluation of the taxonomy of endangered and threatened species. We used the subspecies recognition criteria proposed by Avise and Ball (1990) and O'Brien and Mayr (1991) to examine the infraspecific taxonomy of the leopard,* Panthera pardus, *a geographically widespread species with 27 currently recognized trinomial designations. Samples from named subspecies revealed appreciable genetic diversity using three molecular methods: allozymes, mitochondrial DNA restriction sites, and feline-specific minisatellites. Continental populations and subspecies from Africa and Asia possessed the highest amount of molecular genetic variation, whereas relatively lower amounts of diversity were present in island populations. Molecular data were analyzed using three phylogenetic methods (distance-matrix, maximum parsimony, and maximum likelihood) to resolve genetic differentiation below the species level. The combined results revealed phylogenetic distinction of six geographically isolated groups of leopards: (1) African, (2) central Asian, (3) Indian, (4) Sri Lankan, (5) Javan, and (6) east Asian. Based on the combined molecular analyses and supporting morphological data (Mithtbapala 1992), we recommend that subspecific leopard taxonomy be revised to comprise eight subspecies: (1)* P. p. pardus, *Africa; (2)* P. p. saxicolor, *central Asia; (3)* P. p. fusca, *Indian subcontinent; (4)* P. p. kotiya, *Sri Lanka; (5)* P. p. melas, *Java; (6)* P. p. orientalis, *Amur; (7)* P. p. japonensis, *northern China; and (8)* P. p. delacouri, *southern China. In most cases, designated subspecies conform to historic geological barriers that would have facilitated allopatric genetic divergence.*

Reconocimiento Filográfico de Subespecies en Leopardos (*Panthera pardus*): *Variación Genética Molecular*

Resumen: *La incorporación de definiciones precisas para unidades taxonómicas en legislación de la vida silvestre ha necesitado de la re-evaluación de la taxonomía de especies amenazadas y en peligo de extinción. Utilizamos los criterios de reconocimiento de subespecies propuestos por Avise y Ball (1990) y O'Brien y Mayr (1991) para examinar la taxonomía intraespecífica del leopardo* Panthera pardus, *una especie ampliamente dispersa con 27 designaciones trinomiales reconocidas. Muestras de supuestas subespecies revelaron una diversidad genética apreciable, usando tres métodos moleculares: Alozimas, sitios de restricción en ADN mitocondrial y minisatélites felino-específicos. Poblaciones continentales y subespecies de Africa y Asia poseen la más alta cantidad de variación genética molecular, mientras que en poblaciones insulares estuvieron presentes cantidades relativamente bajas de diversidad. Los datos moleculares fueron analizados utilizando tres métodos filogenéticos (Matriz de distancia, máxima parsimonia y máxima proximidad) para resolver diferenciaciones genéticas por debajo del nivel de especie. Los resultados combinados revelaron la distinción filogenética de seis grupos de leopardos geográficamente aislados: 1) Africano, 2) Centro asiático, 3) Hindú, 4) Sri Lankano, 5)* Javano y, *6) Este asiático. Basados en el análisis molecular combinado y soportados en datos morfológicos (Mithtbapala, 1992), recomendamos la revisión taxonómica a nivel de subespecie que com-*

§ *Current address: 176 Polhengoda Road, Colombo 5, Sri Lanka.*
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prende ocho subespecies: 1) P. p. pardus, *Africa; 2)* P. p. saxicolor, *Asia central; 3)* P. p. fusca, *subcontinente Hindú; 4)* P. p. kotiya, *Sri Lanka; 5)* P. p. melas, *Java; 6)* P. p. orientalis, *Amur; 7)* P. p. japonensis, *norte Chino; 8)* P. p. delacouri, *sur Chino. En la mayoría de los casos las subespecies designadas están conformadas por barreras geológicas históricas que pudieron haber facilitado divergencia genética alopátrica.*

Introduction

During the last century, geographic variants of species were named as subspecies without clear definition of the unit. When this indeterminate taxonomy is overlaid with precise wildlife legislation of the late twentieth century, it is sometimes detrimental to conservation efforts (Avise & Nelson 1989; Daugherty et al. 1990; O'Brien & Mayr 1991; Wayne & Jenks 1991). Thus, there is an imminent need for conservation biologists to define taxonomic units explicitly, particularly those that are endangered and threatened (Daugherty et al. 1990).

The definition of subspecies, a taxonomic category below the species level that recognizes geographic and temporal subdivisions (Mayr 1982*a*), has been the subject of academic dispute for decades (Wilson & Brown 1953; Barrowclough 1982; Gill 1982; Mayr 1982*b*; Parkes 1982). With the emergence of conservation biology as a science and the incorporation of subspecies into the U.S. Endangered Species Act (U.S. Fish and Wildlife Service 1973), clear delineation of this category became essential. Recently, molecular biologists involved with the conservation of endangered species have attempted to incorporate genetic differentiation into the definition of a subspecies (Avise & Ball 1990; O'Brien & Mayr 1991), although controversy continues over the best approach to recognition of species and subspecies (Otte & Endler 1989; Amato 1991; Geist 1992). In this study of leopard populations we employ the general definition proposed by O'Brien and Mayr (1991) that members of a subspecies would "share a unique geographic locale, a set of phylogenetically concordant phenotypic characters, and a unique natural history relative to other subdivisions of the species. Although subspecies are not reproductively isolated, they will normally be allopatric and exhibit recognizable phylogenetic partitioning." Furthermore, "evidence for phylogenetic distinction must normally come from the concordant distributions of multiple, independent genetically based traits" (Avise & Ball 1990).

The geographic distribution of leopards (*Panthera pardus*) extends throughout Africa, central Asia, southeast Asia, and north to the Amur Valley in Russia (Fig. 1). Twenty-seven leopard subspecies have been described based on phenotypic and geographical variation (Table 1). Three populations living on the islands of Java, Sri Lanka, and Zanzibar have been designated as distinct subspecies. Several subspecific designations were based upon a few skins or skull specimens (Pocock 1932; Zukowsky 1964), raising serious questions about their taxonomic distinctiveness (Neff 1983). Our study examines phylogeographic genetic divergence among leopard subspecies based upon three categories of molecular genetic markers: mitochondrial DNA restriction fragment length polymorphism (mtDNA-RFLP), nuclear allozyme locus variation, and nuclear minisatellite (or variable number tandem repeat, VNTR) variation. These markers have been used successfully in taxonomic applications relevant to conservation (Avise & Nelson 1989; Gilbert et al. 1990; O'Brien et al. 1990; Wayne & Jenks 1991; Avise 1994; O'Brien 1994*a*,1994*b*).

Subspecies recognition and verification were approached in two steps. First, phylogenetic analyses of differentiation for each gene family were used to resolve natural genetic distinctions that developed over evolutionary time. Recognizable phylogenetic clusters (or clades) were identified using three analytical methods: (1) phenetic or genetic distance matrix-based topologies; (2) maximum parsimony; and (3) maximum likelihood. Second, the recognized phylogenetic population clusters were verified by identification of the molecular genetic markers that contributed to phylogenetic distinction. The greatest weight was given to genetically fixed characters (Avise & Ball 1990; O'Brien & Mayr 1991), followed by population-specific alleles, restriction sites, or haplotypes. The least weight was given to divergent allele frequencies in separate populations.

Methods

Total genomic DNA isolated from leukocytes or tissue samples was digested with a panel of 30 restriction enzymes: *AccI, ApaI, AvaI, AvaII, BamHI, BclI, BglI, BglII, BstEI, BstUI, ClaI, DraI, EcoRI, EcoRV, HincII, HindIII, HpaI, HpaII, KpnI, NcoI, NdeI, PstI, PvuI, PvuII, SalI, SstII, StuI, XbaI,* and *XhoI* (Bethesda Research Laboratories and New England Biolabs). Digested samples were electrophoresed, transferred to nylon membranes (UV Duralon, Stratagene), and hybridized to random-primed, cloned mtDNA from the domestic cat, as previously described (O'Brien et al. 1990; Menotti-Raymond & O'Brien 1993). Restriction fragment length polymorphisms, RFLP, were visualized by autoradiography. Mitochondrial DNA diversity was estimated between populations, d_a, and within populations, π (Nei & Li 1979),

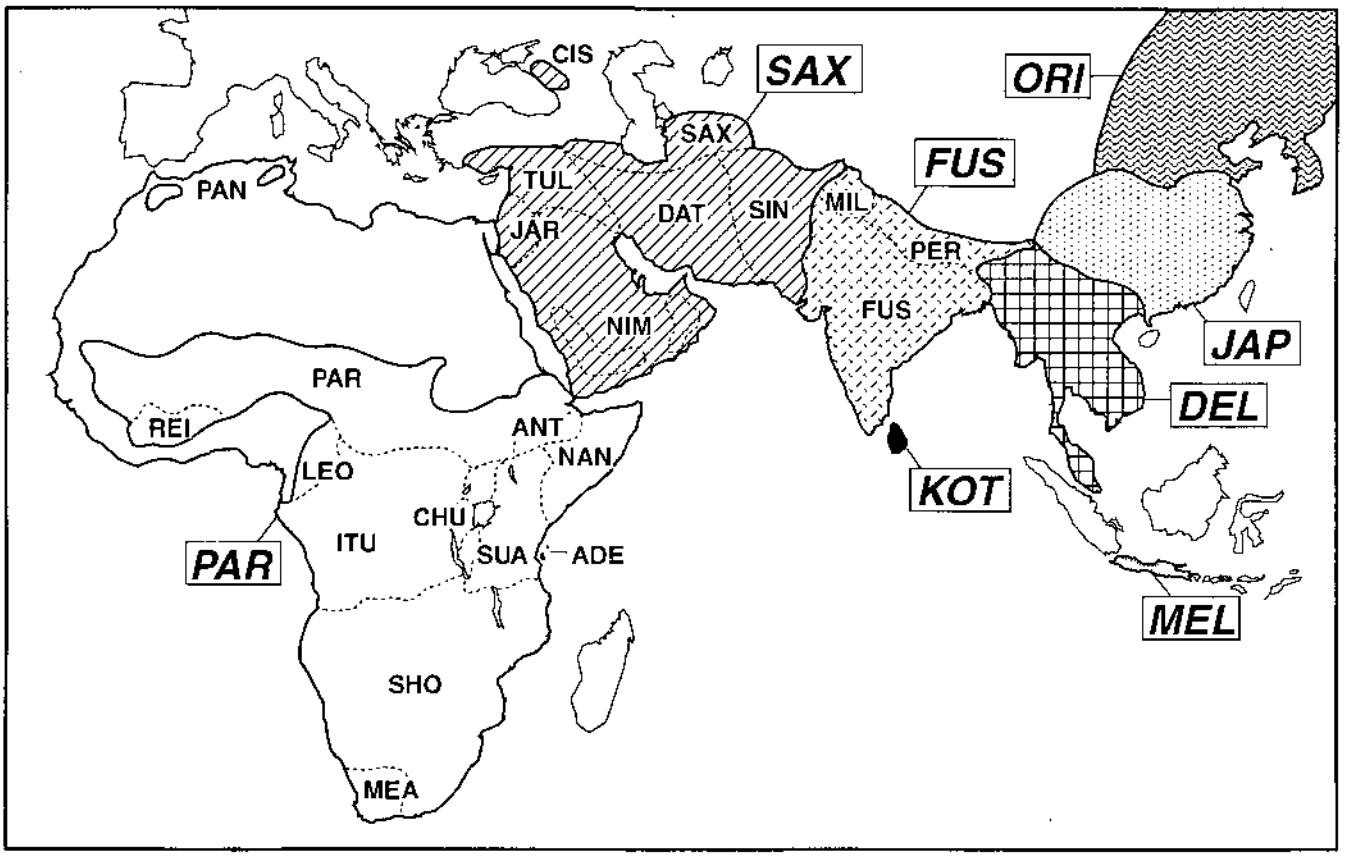

Figure 1. Range of 27 named subspecies of leopards. Three-letter code designations are listed in Table 1. Differential hatch and boxed codes indicate geographic ranges of the eight revised subspecies defined by this analysis. Sub-Saharan leopard populations have been isolated from northern populations by the desertification of the Sahara, which formed a barrier to dispersal during the late Pleistocene, some 20,000 years ago (Maglio 1978). Java and Sri Lanka were probably last joined to their respective mainlands, Malaysia and India, at the end of the late Pleistocene (Jacob 1949; Seidensticker 1986), and hence these insular populations have been distinct for some 10,000 years.

using maximum-likelihood estimates computed by the MAXLIKE program (Nei & Tajima 1983).

Allozyme electrophoresis for 49 gene-enzyme loci was performed with extracts from erythrocytes, leukocytes, plasma, and tissue-culture fibroblast cells using standard protocols (Harris & Hopkinson 1976; O'Brien 1980; Newman et al. 1985; Miththapala 1992). Allelic frequencies for each allozyme locus were estimated from individual genotypes and used to calculate Nei's unbiased genetic distance (Nei 1978) between all pairs of species examined using BIOSYS-1 (Swofford & Selander 1981). Nei's (1987) test of significance (with a Bonferroni adjustment to correct for the violation of the assumption of independence) was used to examine differences between average heterozygosities.

Nuclear minisatellite (also called VNTR or DNA fingerprints) variation was determined using feline-specific FCZ8 and FCZ9 minisatellite clones (Gilbert et al. 1991; Menotti-Raymond & O'Brien 1993) and was quantified as average percent difference (APD) in band-sharing plus estimated average heterozygosity (Miththapala 1992; Stephens et al. 1992). Genomic DNA of the *P. p. delacouri* and *P. p. suahelicus* samples were degraded and excluded from this analysis.

Phenograms describing the associations among the species were constructed from the distance matrices for each gene family using the Fitch-Margoliash algorithm (FITCH and KITSCH) of PHYLIP version 3.4 (Felsenstein 1991). Character data for allozymes, mtDNA restriction sites, and VNTR fragments were generated for each species by transforming allelic frequencies into discrete character states: each polymorphic allele was coded as a discrete character and scored for presence or absence in each individual, subspecies, or population. Character data were analyzed by maximum parsimony using the program PAUP version 3.0 (Swofford 1991) and, for

Table 1. Traditional and revised subspecific taxonomy of *Panthera pardus*.

Common name	*Traditional subspecies*	*Codes*[a]	*USFW*[b]	*TAG*[c]	*No. samples (wild-caught)*		*Revised subspecies*
					Molecular	*Morphological*	
1. Barbary leopard	*P. p. panthera* (Schreber 1777)	PAN	E	C	2 (1)	3	*P. p. pardus*
2. North African leopard	*P. p. pardus* (Linneaus 1758)	PAR	E	E	—	0	*P. p. pardus*
3. Eritrean leopard	*P. p. antinorii* (de Beaux 1923)	ANT	E	S	—	0	*P. p. pardus*
4. West African leopard	*P. p. reichenowi* (Cabrera 1918)	REI	T	V	2 (2)	6	*P. p. pardus*
5. West African forest leopard	*P. p. leopardus* (Schreber 1777)	LEO	E	V	—	11	*P. p. pardus*
6. Central African leopard	*P. p. shortridgei* (Pocock 1932)	SHO	T	S	11 (9)	43[d]	*P. p. pardus*
7. Cape leopard	*P. p. melanotica* (Gunther 1885)	MEA	T	S	—	0	*P. p. pardus*
8. East African leopard	*P. p. suahelicus* (Neumann 1900)	SUA	T	S	1 (1)	64	*P. p. pardus*
9. Zanzibar leopard	*P. p. adersi* (Pocock 1932)	ADE	T	C	—	1[d]	*P. p. pardus*
10. Ugandan leopard	*P. p. chui* (Heller 1913)	CHU	T	S	—	0	*P. p. pardus*
11. Congo leopard	*P. p. iturensis* (Allen 1924)	ITU	T	S	—	19	*P. p. pardus*
12. Somalian leopard	*P. p. nanopardus* (Thomas 1904)	NAN	E	C	—	4[d]	*P. p. pardus*
13. Asia Minor leopard	*P. p. tulliana* (Valenciennes 1856)	TUL	E	C	—	1	*P. p. saxicolor*
14. Sinai leopard	*P. p. jarvisi* (Pocock 1932)	JAR	E	EX	—	1[d]	*P. p. saxicolor*
15. South Arabian leopard	*P. p. nimr* (Ehrenberg & Hemprich 1833[e])	NIM	E	C	—	1	*P. p. saxicolor*
16. Caucasus leopard	*P. p. ciscaucasicus* (Satunin 1914)	CIS	E	C	—	0	*P. p. saxicolor*
17. Central Persian leopard	*P. p. dathei* (Zukowsky 1964)	DAT	E	C	—	0	*P. p. saxicolor*
18. North Persian leopard	*P. p. saxicolor* (Pocock 1927)	SAX	E	E	12	5[d]	*P. p. saxicolor*
19. Baluchistan leopard	*P. p. sindica* (Pocock 1930*a*)	SIN	E	E	1 (1)	1[d]	*P. p. saxicolor*
20. Kashmir leopard	*P. p. millardi* (Pocock 1930*b*)	MIL	E	V	—	2[d]	*P. p. fusca*
21. Nepal leopard	*P. p. pernigra* (Hodgson 1863[f])	PER	E	V	—	5	*P. p. fusca*
22. Indian leopard	*P. p. fusca* (Meyer 1794[g])	FUS	E	V	3 (3)	64	*P. p. fusca*
23. Sri Lankan leopard	*P. p. kotiya* (Deraniyagala 1956)	KOT	E	C	22 (7)	29	*P. p. kotiya*
24. South Chinese leopard	*P. p. delacouri* (Pocock 1930*b*)	DEL	E	V	5	1[d]	*P. p. delacouri*
25. Amur leopard	*P. p. orientalis* (Schlegel 1857)	ORI	E	C	23	1[d]	*P. p. orientalis*
26. North Chinese leopard	*P. p. japonensis* (Gray 1862)	JAP	E	E	12	4[d]	*P. p. japonensis*
27. Javan leopard	*P. p. melas* (Cuvier 1809)	MEL	E	C	1/95	12	*P. p. melas*

[a] *Code: each subspecies has been assigned a three-letter code that is used throughout this paper. Tissue sample sources:* **PAN** *— M. Bleyman, Carnivore Preservation Trust, Pittsboro, North Carolina;* **REI, SHO-Z, SUA** *— V. Wilson, Chipangali Wildlife Trust, Zimbabwe;* **SHO-SA** *— V. De Vos, Kruger National Park, South Africa;* **SAX** *— D. Gillespie, Cincinnati Zoo, Ohio; D. Hansbury, Lowry Park Zoo, Tampa, Florida; L. Machado, San Francisco Zoo, California; L. Goltenboth, Wilhelmina Zoo, Berlin, Germany; L. Dittrich, Hannover Zoo, Germany; O. Behlert, Koln Zoo, Cologne, Germany; A. Greenwood, Welsh Mountain Zoo, Colwyn Bay, Wales, United Kingdom; D. Jauch, Stuttgart Zoo, Germany;* **SIN** *— T. Meehan, Lincoln Park Zoo, Chicago, Illinois;* **FUS** *— U. Karanth, Nagarahole National Park, Nagarahole, India;* **KOT, DEL** *— S. B. U. Fernado, National Zoological Gardens, Dehiwela, Sri Lanka;* **MEL** *— L. Goltenboth, Wilhelmina Zoo, Berlin, Germany;* **JAP** *— D. Armstrong, Henry Doorly Zoo, Omaha, W. De Meurichy, Antwerp Zoo, Belgium;* **ORI** *— J. Bircher, St. Louis Zoo, Missouri; J. Wortman, Denver Zoo, Colorado; J. M. Lernould, Mulhouse Zoo, France; R. Faust, Frankfurt Zoo, Germany.*

[b] *USFW: U. S. Fish & Wildlife Service; E: endangered, in danger of extinction; T: threatened, likely to become endangered within the foreseeable future. The Convention for International Trade in Endangered Species of Wild Flora and Fauna (CITES) lists all* Panthera pardus *subspecies in Appendix I, i.e., commercial trade is prohibited. But, hunting quotas are permitted in Botswana, Malawi, Namibia, Zambia, and Zimbabwe (U. S. Fish and Wildlife Federal Register Notice 1992).*

[c] *TAG: Felid Action Plan 1992, based on Mace & Lande (1990); C: critical, 50% probability of extinction within 5 years or 2 generations, whichever is longer; E: endangered, 20% probability of extinction within 20 years or 10 generations, whichever is longer; V: vulnerable, 10% probability of extinction within the next 100 years; S: safe, no danger of extinction; EX: extinct.*

[d] *Type specimen included.*

[e] *In Harrison 1968.*

[f] *In von Dobroruka 1964.*

[g] *In Pocock 1930b.*

mtDNA RFLP, by a maximum-likelihood algorithm available in RESTML of PHYLIP version 3.4 (Felsenstein 1991).

Results

Mitochondrial DNA

Cellular DNA from 60 unrelated leopards representing 12 named subspecies (Table 1) were sampled using 30 restriction enzymes. A total of 97 restriction sites was scored, representing 539 base pairs, or 3.3%, of the 16,500 base-pair mtDNA genome. Forty-six sites were variable, producing a total of 18 haplotypes distributed among subspecies as depicted in Table 2.

The amount of mtDNA nucleotide diversity (Table 3) represented in leopards is large (π = 1.29%), surpassing similar estimates for cheetahs (π = 0.182%), giant pandas (π = 0.27%), humans (π = 0.57%), and humpback whales (π = 0.25%) by several-fold (Wilson et al. 1985;

Table 2. Mitochondrial DNA haplotypes and nucleomorphs for polymorphic restriction enzymes.[a]

AvaI	*AvaII*	*BstUI*	*ClaI*	*EcoRV*	*HincII*	*HindIII*	*HpaI*	*HpaII*	*NcoI*	*NdeI*	*PstI*	*StuI*	*XbaI*	*Haplotype*	*No. of Individuals*	*Subspecies*	*Subspecies group*
B	B	B	B	A	A	A	A	A	E	A	B	B	A	C	4	SHO-Z	I - Africa
B	B	B	B	A	A	A	A	A	D	A	B	B	A	D	1/2/2	SHO-Z/SHO-SA/REI	
C	A	B	A	A	A	A	A	A	G	A	B	B	A	E	1/2	SHO-Z/SHO-SA	
D	B	B	B	A	A	A	A	A	D	A	B	B	A	F	1	PAN	
A	D	B	A	A	C	A	B	A	C	A	A	A	A	A	1/7	SIN/SAX	
A	D	B	A	A	C	A	B	A	C	A	B	A	A	B	1	SAX	II - Central Asia
A	E	B	C	A	C	A	B	A	A	B	B	D	C	G	2	FUS	III - India
A	E	B	C	A	A	A	A	A	A	A	B	A	C	H	1	FUS	
A	E	A	C	A	A	A	A	A	A	A	B	A	A	I	12/1	KOT/DEL[b]	IV - Sri Lanka
A	C	B	C	B	B	A	A	A	F	C	B	C	A	L	1	MEL	V - Java
A	E	B	C	A	A	A	A	B	A	A	B	A	A	J	1	DEL	VI - E. Asia
A	E	B	C	A	A	B	A	B	A	A	B	A	B	M	2	ORI	
A	E	B	C	A	A	A	A	B	B	A	B	A	C	O	5	ORI	
A	E	B	C	A	A	A	A	B	A	A	B	A	B	P	1	ORI	
A	E	B	C	A	A	B	A	B	B	A	B	A	C	Q	1	ORI	
A	E	B	C	A	A	A	A	B	A	B	B	A	B	R	3	JAP	
A	E	B	C	A	A	B	A	B	A	B	B	A	B	S	1	ORI/JAP	
D	E	B	C	A	A	A	A	C	A	B	B	A	C	T	1	JAP	

[a] *Nucleomorphs represent restriction enzyme fragment size (kb) patterns as follows:* AvaI-*A: 7.3, 5.3, 3.8, 2.9; -B: 3.8, 3.5, 3.5, 2.9, 2.9; -C: 12.0, 3.8, 3.5; -D: 8.3, 3.8, 3.5, 2.9, 0.8.* AvaII-*A: 8.2, 5.1, 1.5, 1.0, 1.0; -B: 8.2, 3.2, 2.2, 1.2; -C: 10.1, 2.7; -D: 7.6, 4.0, 1.2, 1.1, 0.8; -E: 7.6, 3.2, 1.6, 1.2, 1.2.* BstUI-*A: 14.1; -B: 11.0, 2.6, 0.5.* ClaI-*A: 14.0; -B: 8.4, 6.1; -C: 6.6, 1.5.* EcoRV-*A: 17.0; -B: 11.8, 7.4.* HincII-*A: 6.7, 3.1, 2.7, 2.2, 1.1; -B: 4.1, 3.1, 2.7, 2.2, 1.9, 1.1, 0.8; -C: 5.3, 3.1, 2.7, 2.2, 1.5, 1.1.* HindIII-*A: 9.9, 5.9; -B: 9.9, 3.6 1.6.* HpaI-*A: 10.8, 3.2, 2.7; -B: 9.1, 3.2, 2.7, 1.6.* HpaII-*A: 8.2, 2.2, 1.8, 1.4, 1.3, 1.2, 1.0, 0.9, 0.5; -B: 8.2, 2.1, 1.8, 1.4, 1.3, 1.2, 1.0, 0.9, 0.5, 0.1; -C: 8.2, 2.1, 1.8, 1.4, 1.2, 1.0, 0.9, 0.6, 0.6, 0.5.* NcoI-*A: 5.7, 5.0, 4.7, 1.4, 1.4, 0.3; -B: 11.8, 5.0, 1.4, 1.4; -C: 11.8, 4.7, 1.4, 1.4, 0.3; -D: 5.7, 4.7, 4.5, 3.7, 1.2; -E: 5.7, 4.7, 3.7, 3.0, 1.5, 1.2; -F: 5.7, 5.0, 4.7, 1.4, 1.4, 1.1, 0.3, 0.3; -G: 5.7, 4.7, 4.5, 4.3, 0.6.* NdeI-*A: 13.5, 5.9; -B: 8.7, 5.9, 4.5; -C: 10.0, 5.9, 1.5, 1.5.* PstI-*A: 9.7, 8.8, 3.4; -B: 9.7, 4.9, 4.1.* StuI-*A: 5.7, 5.1, 4.6, 2.4, 0.5, 0.5; -B: 8.3, 4.0, 4.0, 1.9; -C: 8.3, 4.0, 3.0, 1.9, 1.6; -D: 5.7, 5.1, 3.0, 2.4, 1.4, 0.5, 0.5.* XbaI-*A: 14.4, 1.7, 1.2; -B: 8.9, 5.8, 1.7, 1.2, 0.7; -C: 9.6, 5.8, 1.7, 1.2. The following restriction enyzmes were monomorphic in all leopards:* AccI, ApaI, BamHI, BclI, BglI, BglII, BstEI, DraI, EcoRI, KpnI, PvuI, PvuII, SalI, SstI, SstII, XhoI.

[b] *The occurrence of the haplotype* I *in* P. p. delacouri *is likely a result of a breeding mix-up at the National Zoological Gardens in Sri Lanka. All* P.p. kotiya *samples had a single haplotype I. The* P. p. delacouri *leopards had been deliberately inbred (Mitbthapala et al. 1991). Four had haplotype* J *and the fifth had the* kotiya *haplotype* I. *The first four had the same parents; the fifth did not.*

Table 3. Estimated allozyme and mitochondrial DNA variation (%) within leopard subspecies.

Geographic origin	Code	Allozymes		mtDNA		Geographic subspecies	
		No. of individuals	P[a]	H[b]	No. of individuals	Haplotypes	π (SD)
Zimbabwe	SHO-Z	8	16.3	5.4	6	3	0.50 (0.30)
South Africa	SHO-SA	3	14.3	6.8	3	2	0.90 (0.43)
Kenya	SUA	1	6.1	6.1	—	—	—
Morocco	PAN	2	4.1	2.7	1	1	
Liberia	REI	2	4.1	2.4	2	1	0.00 (0.00)
I. Africa	SHO, SUA, PAN, REI	16	18.4	6.5	12	4	0.66 (0.16)
Asia Minor	SAX	12	8.2	3.3	8	2	0.07 (0.05)
Afghan/Pakistan	SIN	1	2.0	2.0	1	1	—
II. Central Asia	SAX, SIN	13	10.2	3.2	9	2	0.07 (0.05)
III. India	FUS	3	10.2	4.8	3	2	0.25 (0.12)
IV. Sri Lanka	KOT	22	4.1	1.3	12	1	0.00 (0.00)
V. Java	MEL	1	2.0	2.0	1	1	—
Malaysia	DEL	5	4.1	2.0	2	2	0.19 (0.10)
Amur Region	ORI	23	8.2	2.8	10	5	0.21 (0.04)
Northern China	JAP	12	4.1	1.3	5	3	0.05 (0.03)
VI. East Asia	DEL,ORI, JAP	40	10.2	2.3	17	9	0.25 (0.02)
All leopards		95	22.5	2.7	60	18	1.29 (0.0015)

[a] *P= percent polymorphic loci when polymorphism indicates a polymorphic allele frequency of ≥ 5% (Lewontin 1974). Samples of single individuals were not included in variation estimation.*
[b] *H = unbiased average heterozygosity × 100 (Nei 1978).*

Baker et al., 1990; O'Brien et al. 1990; Menotti-Raymond & O'Brien 1993). Diversity estimates within subspecies range from a high of 0.90% in *P. p. shortridgei* to 0.0% in *P. p. kotiya*, which is consistent with the latter subspecies' history as an island population (Miththapala et al. 1991). Central Asian subspecies had only two haplotypes and a low diversity estimate, $\pi = 0.07\%$, although this may be a sampling error due to limited sample size ($n = 9$). The highest level of variation, $\pi = 0.66\%$, was observed among the African group.

Genetic divergence in mtDNA between the 12 sampled subspecies was estimated using d_a, the fraction of nucleotide divergence calculated using restriction-site data (Table 4; Nei & Li 1979). An unrooted phenogram based upon the corrected nucleotide distance matrix in Table 4 (using the Fitch-Margoliash tree-building algorithm without the presumption of a molecular clock) is presented in Fig. 2a. The analysis revealed four primary groupings that correspond to geographic origins: (1) African, including REI, PAN, SHO-Z, and SHO-SA; (2) central Asian, including SIN and SAX; (3) Asian, including JAP, ORI, DEL, KOT, and FUS; and (4) Javan, MEL. Imposition of the molecular clock assumption using the KITSCH phenetic algorithm on a restriction-fragment-sharing (without site inference) distance matrix (Fig. 2b) recapitulated the four groupings, with rather deep phy-

Table 4. Percent nucleotide divergence between subspecies, calculated using restriction-site data for mitochondrial DNA.[a]

	SHO-Z[b] (6)	*SHO-SA[b] (3)*	*(2)*	*REI (1)*	*PAN (1)*	*SIN (8)*	*SAX (3)*	*FUS (12)*	*KOT (2)*	*DEL (1)*	*MEL (10)*	*ORI (5)*	*JAP*
SHO-Z[b]	—	0.0	0.04	0.47	2.58	2.53	2.58	2.48	2.37	1.98	2.38	2.71	
SHO-SA[b]		0.59	—	0.00	0.26	2.38	2.16	2.35	2.25	2.14	1.74	2.15	2.48
REI	0.29	0.45	—	0.60	2.78	1.73	2.74	2.44	2.53	2.14	2.54	2.87	
PAN	0.72	0.71	0.60	—	3.05	3.00	3.00	2.91	2.80	2.39	2.80	3.13	
SIN	2.83	2.77	2.78	3.05	—	0.00	0.76	0.91	0.81	2.39	0.98	1.16	
SAX	2.82	2.75	3.03	0.87	0.03	—	0.71	0.85	0.74	2.31	0.92	1.10	
FUS	2.96	2.93	2.87	3.13	0.89	0.89	—	0.32	0.22	1.93	0.30	0.24	
KOT	2.73	2.71	2.64	2.91	0.91	0.88	0.45	—	0.00	1.82	0.39	0.45	
DEL	2.72	2.70	2.63	2.89	0.91	0.88	0.45	0.09	—	1.72	0.19	0.25	
MEL	2.23	2.30	2.14	2.39	2.39	2.34	2.05	1.82	1.82	—	2.01	2.05	
ORI	2.74	2.71	2.65	2.90	1.08	1.06	0.54	0.50	0.40	2.11	—	0.16	
JAP	3.00	2.97	2.91	3.17	1.20	1.16	0.41	0.48	0.39	2.09	0.30	—	

[a] *Above diagonal: d_a, corrected for within-population diversity; below diagonal: d_{xy}, uncorrected for within-population diversity. Subspecies codes are given in Table 1; number of specimens typed are given in parentheses.*
[b] *Two populations of* shortridgei *were sampled from Zimbabwe and from South Africa.*

logenetic nodes supporting the monophyly of the four groups.

Coding the RFLP site variation as discrete phylogenetic characters allowed the analysis of the mtDNA variation using a maximum-parsimony algorithm of the PAUP computer package. More than 50 equally parsimonious bifurcating trees were obtained. A strict consensus tree of derived haplotypes resolved the same four major groupings as the phenetic analysis did, although unresolved polytomies were apparent within the major population clusters (Fig. 2c). Bootstrap resampling of the restriction-site data provided strong support (86–100%) for three of the haplotype clusters: (1) African; (2) Central Asian; and (3) Javan, but less support for the east Asian clade (18% bootstrap replications). When restriction fragment (as opposed to specific restriction sites) variation sharing for each named subspecies was analyzed by PAUP using an outgroup taxon, *Panthera tigris* (tiger), support for four clades was obtained (Fig. 2d).

Different phylogenetic methods were not in agreement on the position of *P. p. fusca*, the Indian subspecies, raising the possibility of a distinct subspecies lineage. With an outgroup taxon, bootstrap support for African and central Asian clades was strong (99% and 90% respectively), but less so for the east Asian (38%), Indian (45%), and Javan (50%) clades (Fig. 2d). Phylogenetic analysis of the 18 mtDNA haplotypes using the maximum likelihood algorithm (Fig. 2e) produced highly significant ($p \leq 0.01$) divergence nodes supporting central Asian, African, Javan, and Indian clusters. East Asian monophyly was not supported because certain Asian haplotypes appeared closer to the African cluster than to other east Asian haplotypes (Fig. 2e).

Allozymes

Of 49 allozyme loci typed, 11 were polymorphic in a sample of 95 leopards from 12 named subspecies (Table 5). There were no fixed allelic differences between populations, although several alleles appeared specific to subspecies groups implicated by the mtDNA analysis. There were six allozyme alleles unique to Africa (*CA2*-a, *HBB*-d, *ITP*-b, *ITP*-c, *PGD*-c, and *TF*-a), one unique to central Asia (*HK1*-b), and one unique to India (*PEPB*-b) (Table 5).

A comparison of allozyme average heterozygosity in each leopard population (Table 3) revealed a range of allelic variation, from a low in *P. p. kotiya* ($P = 4.1\%$, $H = 1.3\%$) to a high of $P = 16.3\%$, $H = 5.4\%$ in the Zimbabwe *P. p. shortridgei* population. With the Bonferroni adjustment there were no significant differences between subspecies in estimated average heterozygosities. But the island population from Sri Lanka, *P. p. kotiya*, displayed the lowest amount of variation relative to African, Indian, and other Asian populations. A similar tendency toward low variation was seen in the captive populations, *P. p. japonensis*, *P. p. orientalis*, and *P. p. saxicolor*, suggesting that genetic drift may have reduced variation during captive propagation. One leopard subspecies, *P. p. shortridgei*, had greater variation ($P = 16\%$, $H = 5.4\%$) than outbred, free-ranging African lions ($P = 11\%$, $H = 3.8\%$) and tigers ($P = 10\%$, $H = 3.5\%$), whereas the island population from Sri Lanka, *P. p. kotiya*, had limited variation ($P = 4\%$, $H = 1.3\%$) approaching that of the genetically depauperate populations of cheetah ($P = 2.0\%$, $H = 0.04\%$) and Asian lion ($P = 0.0\%$, $H = 0.0\%$) (O'Brien et al. 1985, 1987; Mitbthapala et al. 1991).

Allozyme genetic distances between subspecies (Table 6) are small, as might be expected within species (Avise & Aquadro 1982; O'Brien et al. 1987), and they therefore produce only modest phylogenetic resolution (Fig. 3a & b). A Fitch-Margoliash phenogram based upon the allozyme genetic distance matrix (Fig. 3a) resolved continental (Asian versus African) distinctiveness; beyond this, little monophyletic clustering is resolved. The character-state analysis based on maximum parsimony (Fig. 3b) supported the African monophyly relative to Asian populations but did not produce further phylogeographic resolution.

VNTR Variation

The extent and pattern of minisatellite, or VNTR, variation in the 12 sampled subspecies were determined using representatives of each of the 18 mtDNA haplotypes from each subspecies. All samples were analyzed for restriction-fragment sharing following digestion with two restriction enzymes, *RsaI* and *HinfI*, and hybridization with two feline-specific minisatellite probes, FCZ8 and FCZ9 (Gilbert et al. 1991; Menotti-Raymond & O'Brien 1993). Genetic variation was assessed by computation of the average percent difference, APD, in band-sharing between individuals and the estimated average heterozygosity (Stephens et al. 1992). Average percent difference and percent average heterozygosity, H, are highly correlated with each other for DNA fingerprinting data ($r = 0.986$) and with other measures of overall genomic variation collected in our laboratory (Yuhki & O'Brien 1990; Gilbert et al. 1990, 1991; Stephens et al. 1992). Leopards display a considerable amount of VNTR variation, and every VNTR fragment scored was polymorphic. In order to maximize representation on gels, only a single individual of each subspecies was sampled, so some of the fixed differences and unique alleles are likely to result from small-sample bias. This strategy was meant to detect a maximum of genetic variation with limited samples but would also overestimate average population and species variability.

A matrix of mean average percent difference (MAPD; Table 6) reveals appreciable divergence within and between subspecies (34.9–82.8% MAPD). A Fitch-Margoliash tree is presented in Fig. 4a, and a PAUP unrooted strict con-

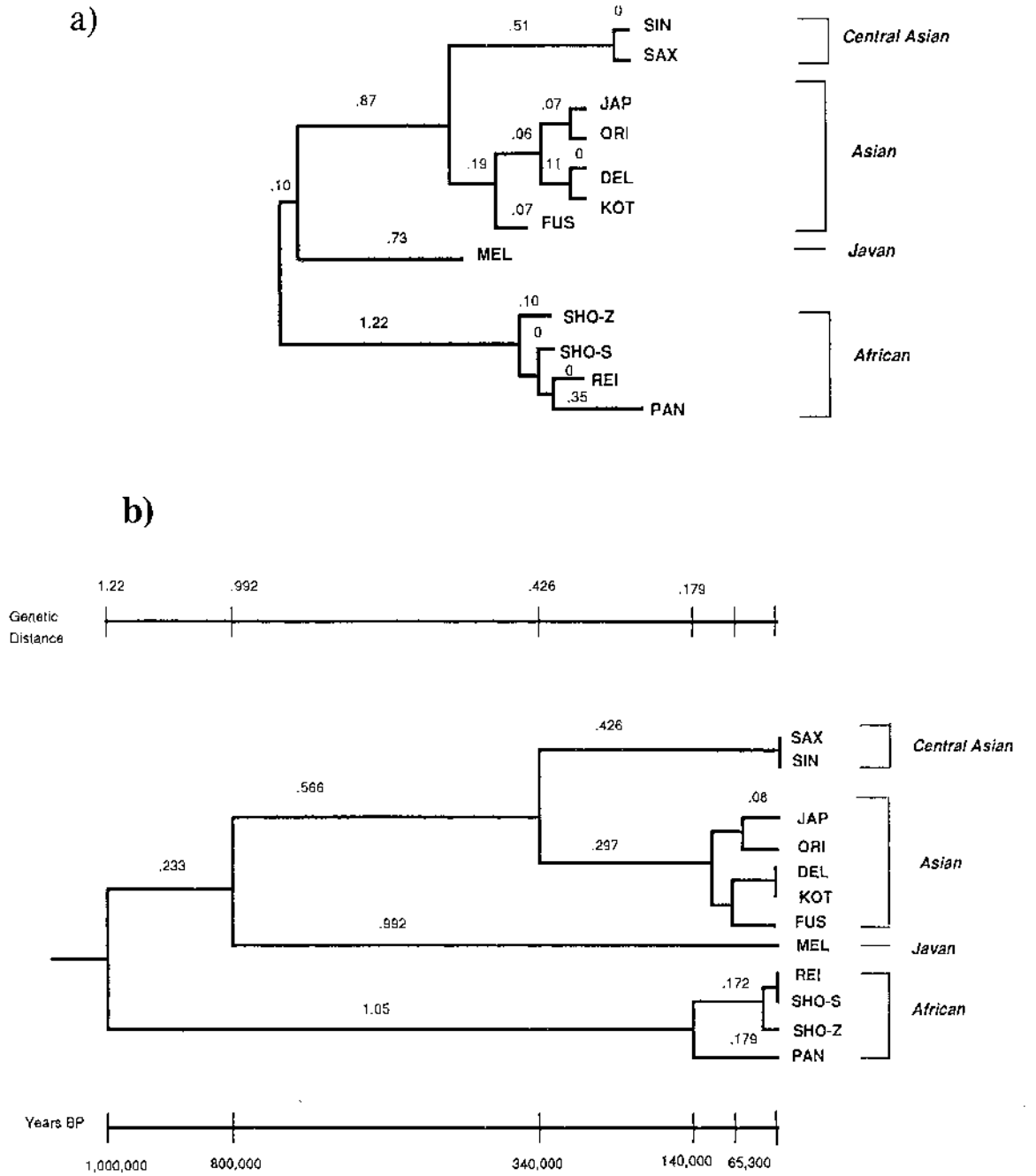

Figure 2. Phylogenetic analyses of mtDNA-RFLP sites and haplotypes: unrooted FITCH tree generated using interpopulation divergences calculated from mtDNA site variation reported in Table 4 (sum of squares = 4.4; average percent deviation = 18.4; limb length units are d_a) (a); rooted KITSCH tree generated using interpopulation divergences calculated from mtDNA site variation reported in Table 4 (sum of squares = 5.5; average percent deviation = 20.5; scale calibration based on rate of mtDNA sequence divergence in the genus Panthera, *for which average mtDNA divergence between five species is 10.4% over an interval of 2 million years [Menotti-Raymond & O'Brien 1993]) (b); and unrooted strict consensus tree for mtDNA site variation produced by maximum-parsimony analysis of haplotypes (letters in parentheses) using PAUP (tree length = 67; consistency index = 77.6%; percentages refer to bootstrap replications [out of 1000] that support the respective grouping; numbers refer to site changes [apomorphies in plain text, homoplasies in parentheses]) (c); strict consensus tree rooted with* Panthera tigris *generated by PAUP using mtDNA fragment variation (tree length = 155; consistency index = 81.9%; percentages refer to bootstrap replications [out of 1000] that support the respective grouping; numbers refer to fragment changes [apomorphies in plain text, homoplasies in parentheses]) (d); maximum-likelihood tree generated using mtDNA site variation (all nodes statistically significant at $p \leq 0.05$ confidence interval; nonsignificant bifurcations collapsed into polytomies; Ln likelihood = −401.62) (e).*

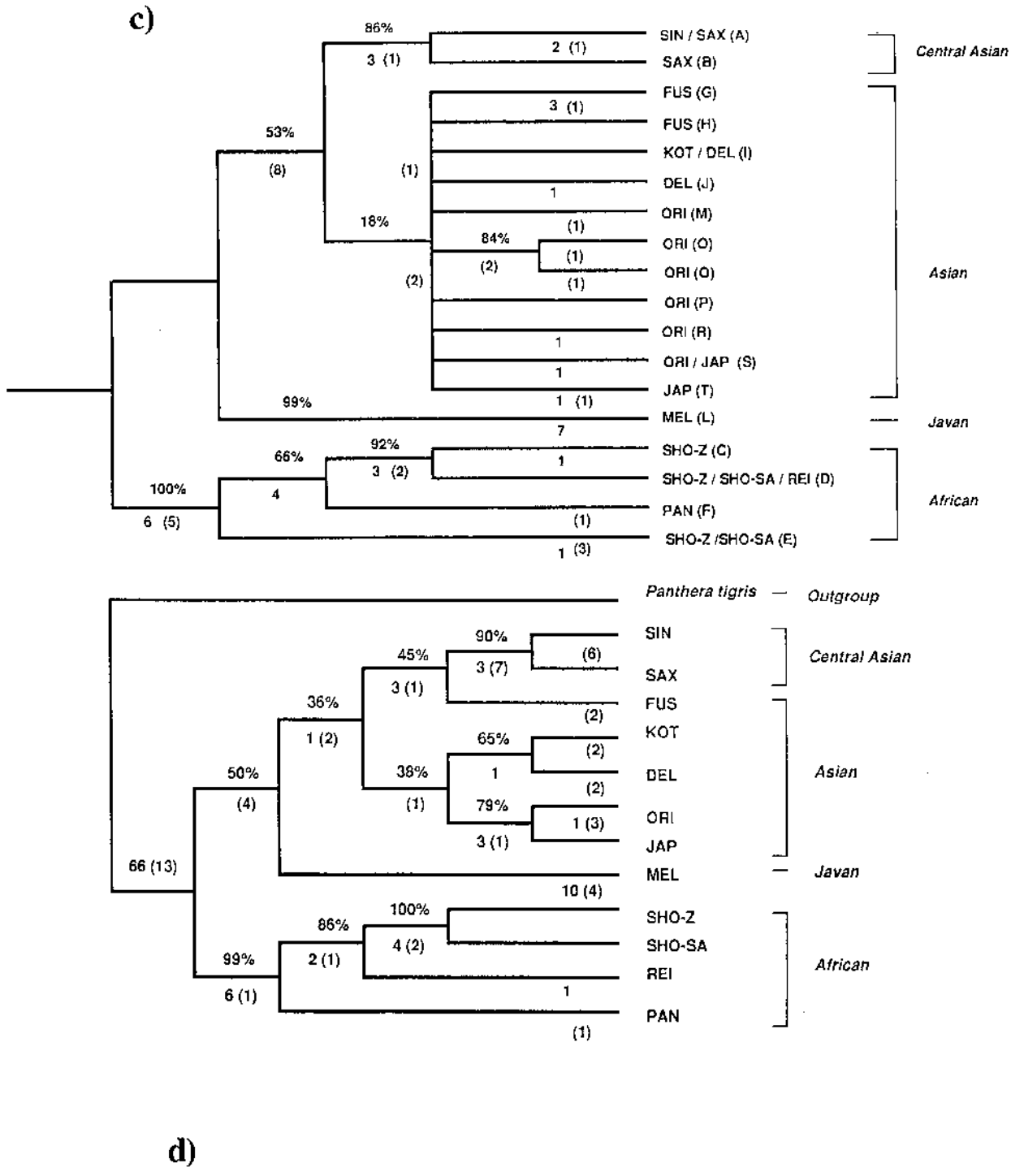

Figure 2. (continued)

sensus tree of fragment characters is presented in Fig. 4b. The VNTR data were consistent with the primary groupings seen with mtDNA and allozyme data. African subspecies clustered together, separate from east Asian and central Asian leopard populations. East Asian and central Asian subspecies were not phylogenetically defined by the VNTR or the allozyme analysis, but, their genetic distinction (detected by the mtDNA data) is not precluded by these two analyses. The VNTR pattern of the *P. p. melas* individual was highly divergent from other Asian genotypes, supporting the recognition of the Javan subspecies from other Asian populations. The consistent support for the major groups (African, east Asian, central Asian, Javan) using different molecular

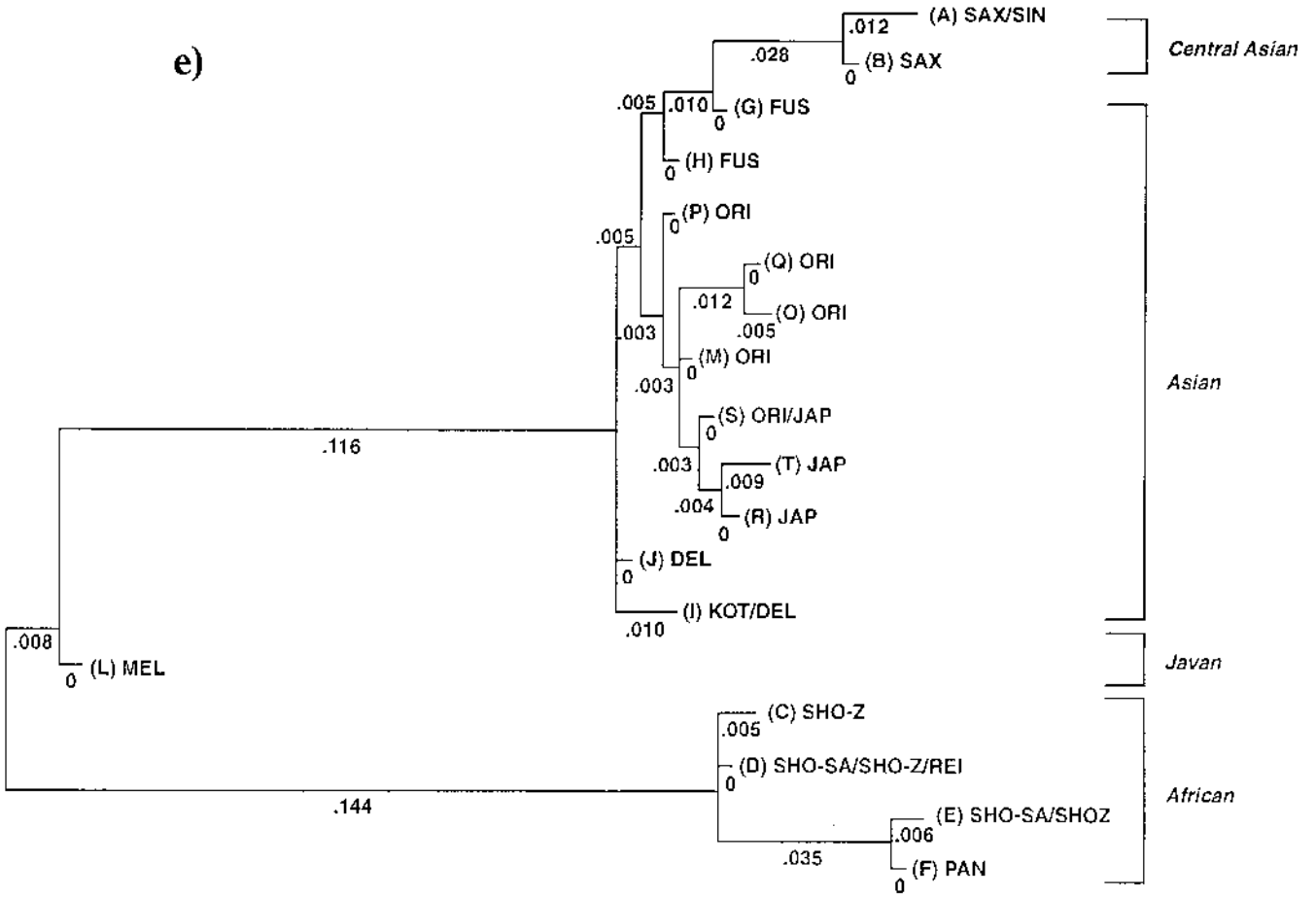

Figure 2. (continued)

methods encourages additional confidence in their recognition as isolated subspecies with identified levels of genetic diversification.

Molecular Phylogenetic Characters that Define Subspecies Differentiation

The composite phylogenetic analyses support the designation of taxonomic units of leopard subspecies that correspond to six geographic locales: Africa, central Asia, India, Sri Lanka, Java, and east Asia (Fig. 1). Table 7 lists the number of identifying molecular characters for each subspecies group. Mitochondrial DNA was the most discriminating metric, with at least one unique site and haplotype specific for each group and up to 10 unique sites in the African subspecies. Allozymes and DNA fingerprints were less informative but provide support in the context of unique polymorphic alleles for each group. Based on these results, we recognize the designation of these six subspecies groups as formal taxonomic units.

Table 8 presents genetic distance matrices between the six identified subspecies using each molecular method. The distances are small, as seen in the phylogenetic analyses, but they do reveal certain notable trends. First, the African subspecies are more divergent from Asian subspecies than Asian subspecies are from each other. Second, the relatively large intercontinental genetic distance plus the larger extent of variation within African populations (Tables 3 and 6) would be consistent with the African lineage being older than those of Asian groups. Third, the two island subspecies, *P. p. kotiya* and *P. p. melas*, have different levels of divergence from their respective adjacent mainland subspecies. *P. p. kotiya* is relatively close to *P. p. fusca* in all measures, whereas *P. p. melas* is very distinct. These quantitative differences imply that *P. p. kotiya* diverged more recently from mainland ancestors, whereas *P. p. melas* represents an earlier departure from mainland ancestors.

Discussion

A molecular genetic analysis of 13 populations of *Panthera pardus* from across its geographical range indicates that extensive infraspecific partitioning supporting 27 subspecies designations (Fig. 1) is inapparent. A group of concordant genetic differences support subspecies recognition (Avise & Ball 1990; O'Brien & Mayr 1991)

Table 5. Allele frequencies of polymorphic allozyme loci[a] among putative leopard subspecies.[b]

Locus	*PAN* (n = 2)	*REI* (n = 2)	*SHO-Z* (n = 8)	*SHO-S* (n = 3)	*SUA* (n = 1)	*SAX* (n = 12)	*SIN* (n = 1)	*FUS* (n = 3)	*KOT* (n = 22)	*DEL* (n = 5)	*MEL* (n = 1)	*JAP* (n = 12)	*ORI* (n = 23)
ADA	a = 0.50	a	a = 0.94	a = 0.17	a	a = 0.59	a	a = 0.83	a	a	a	a = 0.77	a = 0.54
	b = 0.50		b = 0.06	b = 0.83		b = 0.41		b = 0.17				b = 0.23	b = 0.46
APRT	a	a	a = 0.56	a = 0.83	a = 0.50	a = 0.75	a	a = 0.83	a = 0.93	a = 0.40			
			b = 0.44	b = 0.17	b = 0.50	b = 0.25		b = 0.17	b = 0.07	b = 0.60	b	b	b
CA2			a = 0.12[c]										
	b	b	b = 0.88	b	b	b	b	b	b	b	b	b	b
DIA	a	a = 0.50	a = 0.75	a	a	a	a	a	a	a	a	a	a = 0.89
		b = 0.50	b = 0.25								b = 0.11		
ESα-2	a	a	a = 0.79	a = 0.67	a = 0.50	a = 0.67	a	a = 0.67	a	a	a = 0.50	a = 0.91	a = 0.91
			b = 0.21	b = 0.33	b = 0.50	b = 0.33		b = 0.33			b = 0.50	b = 0.09	b = 0.09
HBB	s	s	s = 0.35	s = 0.58		s	s	s	s	s	s	s	s
			d = 0.65	d = 0.42	d								
HK1	a	a	a	a	a	a = 0.96	a	a	a	a	a	a	a
						b = 0.04[c]							
ITP	a	a	a	a = 0.33	a	a	a	a	a	a	a	a	a
			b = 0.50[c]										
				c = 0.17[c]									
PEPB	a	a	a	a	a	a	a	a = 0.67	a	a	a	a	a
								b = 0.33[c]					
PGD	a	a	a = 0.88	a = 0.33		a = 0.92	a = 0.50	a = 0.50	a = 0.48	a = 0.70	a = 0.50	a = 0.96	a = 0.52
					b = 0.08	b = 0.50	b = 0.50	b = 0.52	b = 0.30	b = 0.50	b = 0.04	b = 0.48	
			c = 0.12	c = 0.67	c								
TF	a = 0.50	a = 0.50	a = 0.43	a = 0.17	a = 0.50								
	b = 0.50	b = 0.50	b = 0.57	b = 0.83	b = 0.50	b	b	b	b	b	b	b	b

[a] *Invariant loci included ACP1, ACP2, AK1, ALB, CAT, CPKB, ESU-d, ESα-1, G6PD, GPI, GOT1, GOT2, GPT, GSR, GUSB, GLO, HK2, HEX, HPRT, IDH1, IDH2, LDHA, LDHB, MDH1, MDH2, ME1, MPI, NP, PEPA, PEPC, PEPD, PGAM, PGM1, PGM2, PP, PK, SOD, TPI. Gene symbols are based on homologous human enzyme locus (McAlpine et al. 1991).*

[b] *Subspecies codes are given in Table 1 and* n *is in parentheses.*

[c] *Denotes alleles unique to subspecies.*

Table 6. Molecular genetic distance estimates between leopard subspecies.[a]

Subspecies[b]	*PAN*	*REI*	*SHO-Z*	*SHO-SA*	*SUA*	*SAX*	*SIN*	*FUS*	*KOT*	*DEL*	*MEL*	*JAP*	*ORI*	*PTI*
PAN (1)	—	0.005	0.022	0.025	0.056	0.005	0.012	0.011	0.013	0.015	0.055	0.240	0.028	0.04
REI (2)	64.2	—	0.021	0.049	0.058	0.010	0.009	0.011	0.009	0.012	0.051	0.026	0.032	0.05
SHO-Z (3)	69.7	66.3	—	0.026	0.019	0.027	0.032	0.028	0.032	0.025	0.051	0.031	0.037	0.072
SHO-SA (1)	71.9	60.8	59.0	—	0.027	0.039	0.052	0.043	0.052	0.055	0.075	0.060	0.054	0.079
SUA	—	—	—	—	—	0.052	0.053	0.045	0.052	0.048	0.053	0.057	0.057	0.106
SAX (2)	64.0	77.7	79.5	80.9	—	—	0.010	0.003	0.010	0.008	0.033	0.013	0.016	0.047
SIN (1)	75.3	73.4	78.3	76.5	—	66.6	—	0.002	0.000	0.007	0.031	0.026	0.026	0.054
FUS (2)	73.2	81.2	77.2	76.8	—	65.1	65.9	—	0.001	0.005	0.019	0.018	0.016	0.053
KOT (6)	70.3	71.9	82.0	75.8	—	63.6	69.2	55.5	—	0.006	0.028	0.024	0.023	0.054
DEL	—	—	—	—	—	—	—	—	—	—	0.018	0.005	0.007	0.058
MEL (1)	60.5	75.0	73.7	69.8	—	63.2	68.0	70.8	70.9	—	—	0.023	0.014	0.099
JAP (4)	67.4	74.8	75.7	73.9	—	64.2	62.4	54.7	62.7	—	67.0	—	0.005	0.067
ORI (7)	75.8	69.2	77.7	82.8	—	72.1	71.1	61.7	59.1	—	70.7	34.9	—	0.071

[a] *Above diagonal: unbiased allozyme genetic distance (Nei 1978); below diagonal: mean average percent difference (MAPD) of VNTR band sharing from two minisatellite probes and two restriction enzymes (see Methods). Subspecies codes are given in Table 1; PTI is* Panthera tigris, *tiger. MAPD and estimated average heterozygosity were calculated for four populations with three or more samples: SHO-Z: 59.7 and 44.4; KOT: 31.7 and 30.7; JAP: 33.4 and 28.0; ORI: 35.4 and 34.0.*

[b] *Number in parentheses is number of samples used for minisatellite analysis.*

for six discernible groups: African, central Asian, Indian, Sri Lankan, Javan, and east Asian (Table 7). In contrast, we failed consistently to observe differences between designated African subspecies or between two putative Far Eastern subspecies, *P. p. japonensis* and *P. p. orientalis*. A complementary study of multivariate morphometric analyses of differentiation among all 27 listed subspecies parallels these biochemical results: African, Indian, Sri Lankan, Javan, and Asian specimens were clearly distinct (Miththapala 1992).

The present results provide a basis for a formal revision of the subspecific taxonomy of *P. pardus*. The African populations are clearly distinct from other groups—both qualitatively and quantitatively. But distinctions within Africa are absent: there are shared alleles for allozymes (Table 5), shared mtDNA haplotypes (Table 2), and shared fragments in DNA fingerprints (Table 6). Morphological analysis of cranial characters also failed to reveal significant variation between African subspecies (Miththapala 1992). The combined results argue strongly for subsuming all African populations under one trinomial, *P. p. pardus*, using the principle of priority in taxonomic nomenclature (Wiley 1981).

The Sri Lankan leopard, *P. p. kotiya*, has a unique mtDNA haplotype and unique VNTR fragments, and it is differentiated morphometrically (Table 7). Additional tissue samples from Javan leopards (*P. p. melas*) and wild-caught *P. p. delacouri* from neighboring mainland Malaysia would be required to affirm the existence of a separate clade for *P. p. melas*. Because these populations are naturally insular and allopatric—they have "extrinsic barriers to reproduction" (Avise & Ball 1990)—both these trinomials, *P. p. kotiya* and *P. p. melas*, should be retained.

Tissue samples from the third island subspecies, the Zanzibar leopard, *P. p. adersi*, were not available. Little is known about the natural history and habitat of this leopard, and the delineation of its range and the validity of its subspecific status are controversial (Pakenham 1984). Furthermore, it is now believed to be extinct (T. T. Struhsaker, personal communication).

Of seven putative central Asian subspecies, only two—the Baluchistan leopard, *P. p. sindica*, and the North Persian leopard, *P. p. saxicolor*—were available for molecular analysis. Of these, *P. p. saxicolor* was represented by an entirely zoo-bred population, and there was only a single—albeit wild-caught—sample of *P. p. sindica*. Five subspecies were examined in the morphometric study, but the sample sizes were likely inadequate for firm conclusions (Miththapala 1992). The taxonomy of the remaining central Asian subspecies is tenuous at best: Certain mammalogists consider *P. p. ciscaucasicus* to be synonymous with *P. p. saxicolor* and *P. p. tulliana* (Weinberg, personal communication); *P. p. nimr* was described based on parts of two skins from entirely different locations (Harrison 1968); and *P. p. dathei* was defined based on a single skin (Zukowsky 1964). In addition, the geography of the region lacks major barriers to dispersal. In the light of these facts, and given that *P. p. saxicolor* and *P. p. sindica* share a mtDNA haplotype, all extant central Asian leopards could be subsumed provisionally under *P. p. saxicolor* (Table 1).

There are three putative subspecies on the Indian subcontinent: the Kashmir leopard, *P. p. millardi*; the Nepal leopard, *P. p. pernigra*; and the Indian leopard, *P. p. fusca* (Pocock 1930*b*). Cranial specimens from the Indian subcontinent were morphologically distinguishable as a group, but the type specimens of *P. p. millardi* and *P. p. pernigra* could not be distinguished morphologically from *P. p. fusca* (Miththapala (1992). *P. p. fusca* displays a unique allozyme polymorphism and two distinct mtDNA haplotypes (Tables 2 and 5). Although tissue samples from throughout the subcontinent are neces-

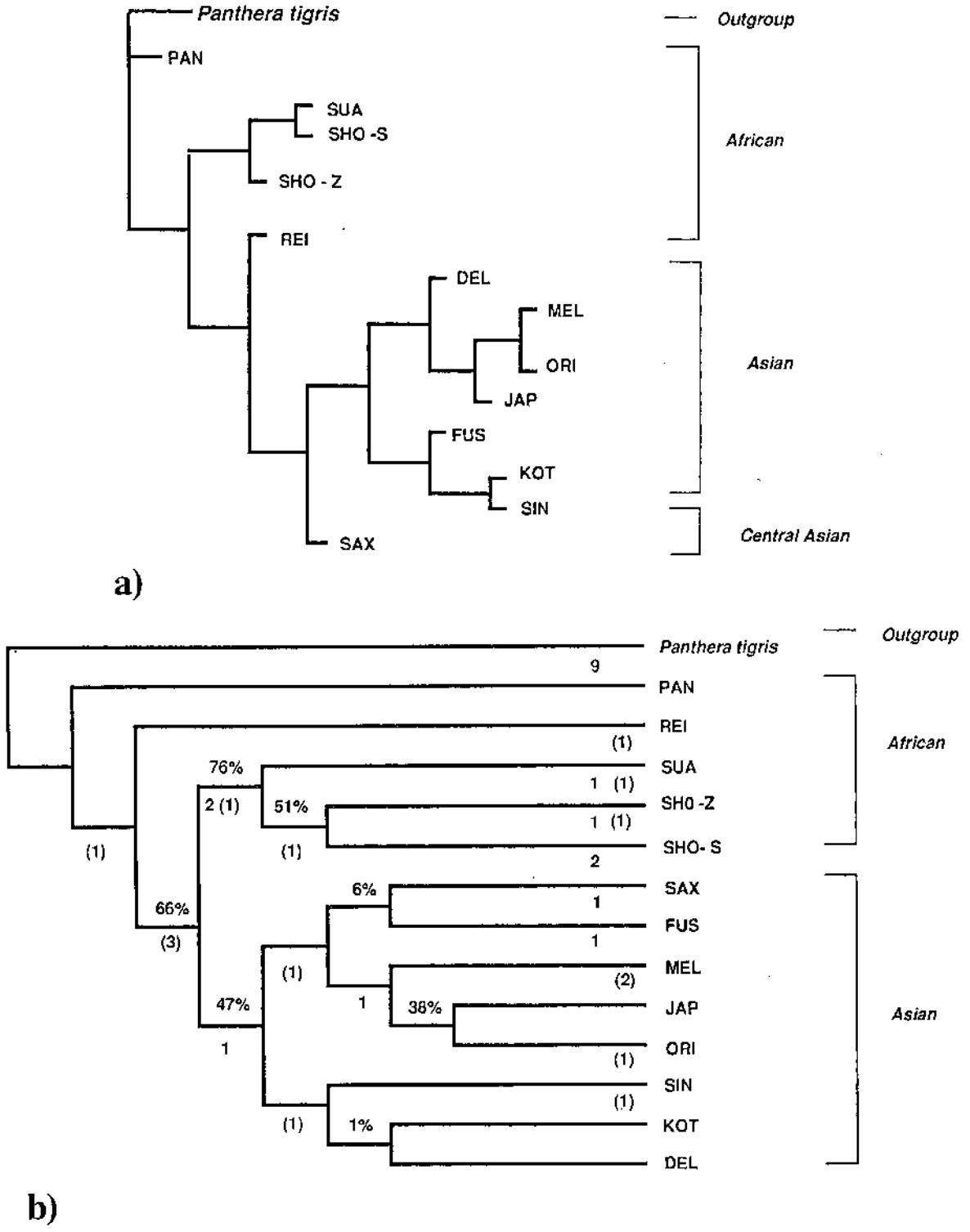

Figure 3. Allozyme phylogenetic analyses: (FITCH trees generated using Nei's [1978] unbiased genetic distances for allozyme data) rooted FITCH tree (with Panthera tigris *as an outgroup) based on unbiased allozyme genetic distance matrix (Table 6) (sum of squares = 7.78; average percent standard deviation = 20.8) (a); rooted strict consensus tree (with* Panthera tigris *as an outgroup) based on maximum-parsimony analysis of allozyme allele characters using PAUP (nine equally parsimonious trees found; tree length = 32; consistency index = 78.1%; percentages refer to the number of bootstrap replications [out of 1000] that support the respective group; numbers refer to character changes [apomorphies in plain text, homoplasies in parentheses]) (b).*

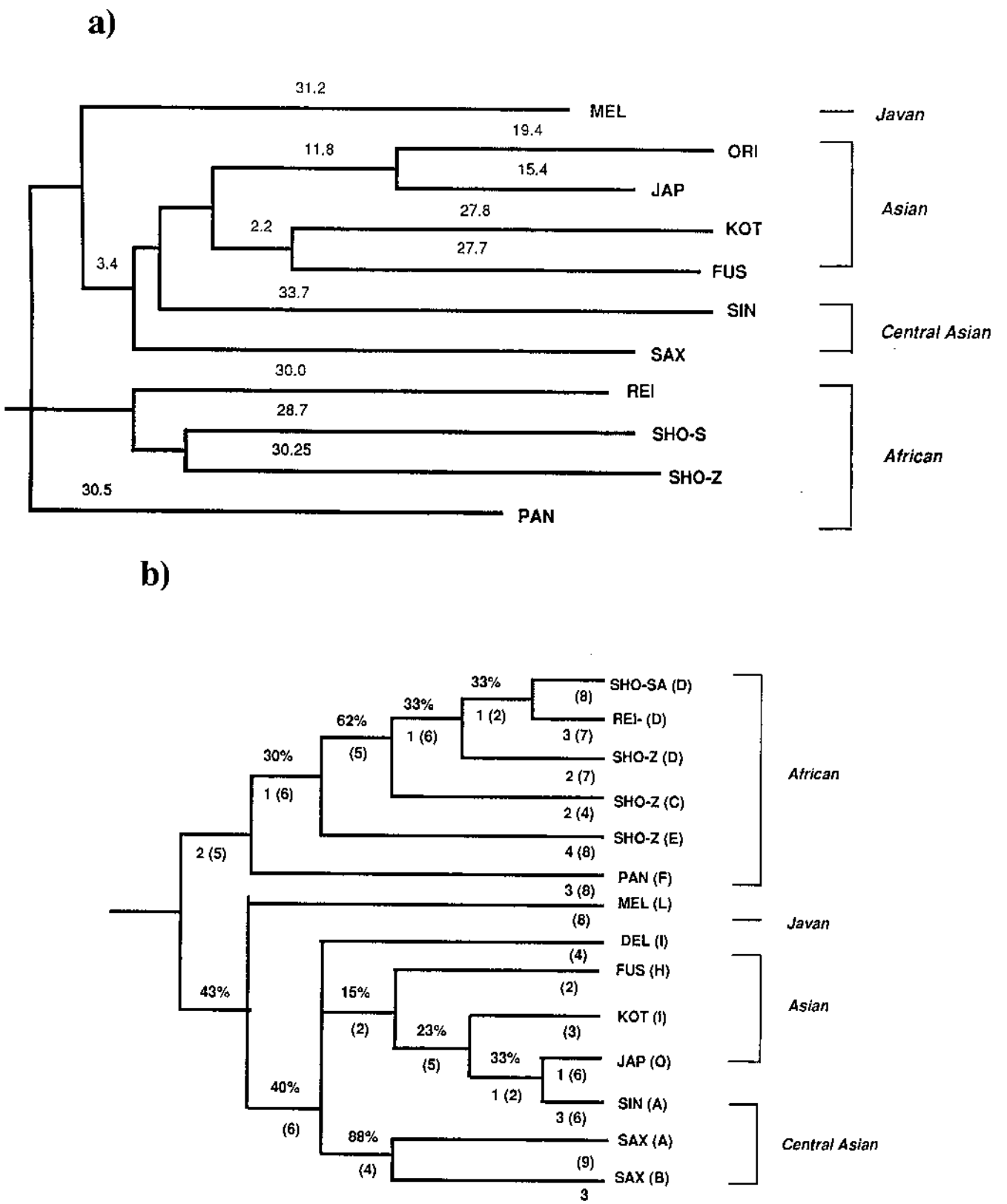

Figure 4. Phylogenetic analyses of minisatellite (VNTR) data: unrooted FITCH tree computed using MAPDs from Table 6 (sum of squares = 0.18; average percent standard deviation = 4.0) (a); unrooted strict consensus tree generated by PAUP for presence or absence of fingerprint fragments for RsaI *and FCZ9 (matrix for these results presented in Miththapala [1992]; two equally parsimonious trees found; tree length = 156; consistency index = 51.9%; percentages refer to the number of bootstrap replications [out of 1000] that support a respective group; numbers in parentheses refer to character homoplasies; letters in parentheses indicate mtDNA haplotype) (b).*

Table 7. Quantified differences among observed subspecies clusters or clades.[a]

Phylogenetic Measure[b]		*I-Africa (16) (92)*	*II-Cen. Asia (13) (3)*	*III-India (3) (33)*	*IV-Sri Lanka (22) (21)*	*V-Java (1) (7)*	*VI-E. Asia (40) (16)*
mtDNA	No. of unique sites	10	3	1	1	6	6
	No. of unique haplotypes	4	2	2	1	1	8
Allozymes	No. of fixed differences	0	0	0	0	0	0
	No. of unique alleles	6	1	1	0	0	0
DNA fingerprinting	No. of unique fragments						
	FCZ 8, RsaI	13	2	0	0	1	3
	FCZ 9, RsaI	8	7	0	0	0	1
	FCZ 8, HinfI	7	5	0	1	7	4
	FCZ 9, HinfI	9	1	0	5	1	0
Morphology	Multivariate differences	+	−	+	+	+	+

[a] *Total number of samples for genetic analysis are given in parentheses on first line; total number of specimens of known sex for morphological analyses are given in parentheses on second line.*
[b] *Specific mtDNA sites and haplotypes specific for subspecies groups are listed in Table 2. Allozyme markers are listed in Table 5. DNA fingerprint fragment data and morphometric analysis of cranial characters are presented in Miththapala (1992).*

sary for confirmation, both molecular and morphological results suggest that the leopards of the Indian subcontinent should be subsumed under the trinomial *P. p. fusca*. Indian topographical features provide a geographic rationale for the assignment, as the Indus River on the west, the Ganges River on the east, and the Himalayas at the north form physical barriers to dispersal.

The south Chinese leopard *P. p. delacouri* was represented in molecular analyses by a deliberately inbred lineage (Miththapala et al. 1991; see Table 2), and the morphometric analysis was performed on a single type specimen (Miththapala 1992). Given these facts, conclusive taxonomic inference should be postponed until further evaluation is possible.

P. p. japonensis and *P. p. orientalis* were paired consistently in phylogenetic analyses (Figs. 2a–d, 3b, 4a) and shared mtDNA haplotypes (Table 2). VNTR divergence between this pair of putative subspecies was low, 34.9%, which is comparable to intrapopulation variation in these two populations: 28.0 and 34%, respectively (Table 6). Allozyme genetic distance between the subspecies is also low (d = 0.005, Table 6). These observations indicate very recent separation, which in turn implies that these populations may not be distinct. Both sampled populations were captive-bred, however, so any taxonomic recommendations must be provisional. Analysis of samples from wild-caught animals of both populations, from different parts of their ranges, is required for further taxonomic resolution. Until then, we recommend retention of the named trinomials.

Management Implications

We reiterate the importance of taxonomy in conservation biology, particularly because precise definitions of

Table 8. Molecular genetic divergence between phylogenetically defined leopard subspecies.[a]

	Central Asia (saxicolor)	*India* (fusca)	*Sri Lanka* (kotiya)	*East Asia*	*Africa* (pardus)	*Java* (melas)
(A)						
Central Asia	—	0.002	0.008	0.012	0.016	0.032
India	7.166	—	0.001	0.014	0.019	0.019
Sri Lanka	0.854	0.325	—	0.019	0.022	0.028
East Asia	0.936	0.245	0.354	—	0.027	0.015
Africa	2.486	2.528	2.433	2.404	—	0.047
Java	2.322	1.927	1.824	1.978	1.929	—
(B)						
Central Asia	—	65.5	66.4	67.4	75.5	65.6
India		—	55.5	58.2	77.1	70.8
Sri Lanka			—	60.9	75.0	76.9
East Asia				—	74.6	68.8
Africa					—	69.7
Java						

[a] *For (A) above diagonal, allozyme genetic distance; below diagonal, π — nucleotide divergence based on mtDNA RFLP. For (B) MAPD for VNTR data. Subspecies names reflect groupings shown in Fig. 1 and Table 1.*

taxonomic units have been incorporated into wildlife legislation (O'Brien & Mayr 1991). We recommend a multidisciplinary approach because our results confirm that modern technological methods—molecular and statistical—allow for quantification of differences and precise characterization of taxonomic units. We suggest that additional geographically widespread species be evaluated taxonomically so that there will be many trinomials to compare. We also recommend that species not yet endangered be examined before conservation challenges become even more difficult.

All three molecular analyses revealed lower levels of heterozygosity and polymorphism in the naturally insular *P. p. kotiya* and in the captive-bred *P. p. japonensis* populations (Tables 3 and 6). Although the other zoo samples of *P. p. orientalis* and *P. p. saxicolor* had higher allozyme variation, all insular populations are tending to fixation at several nuclear loci (Table 5). Therefore, it is important that these zoo populations be managed carefully, with strict avoidance of inbreeding, which will further deplete variation (Chambers 1983). Although the deleterious effects of inbreeding have been recognized (Ralls et al. 1979), breeders of pedigreed leopards still allow consanguineous breeding (Shoemaker 1983, 1988). It is also essential that populations are censused and studied in the wild, as well as sampled for molecular analyses. Comparison of wild samples with zoo samples will allow assessment of the degree of allele fixation due to drift and inbreeding.

Careful monitoring of Sri Lankan and Javan island populations is essential, not only because they are taxonomically distinct but also because they are ecologically significant. On both islands leopards are top carnivores—in Sri Lanka since it separated from India and in Java since the Javan tiger (*Panthera tigris sondaica*) became extinct during the 1970s (Seidensticker 1987). Because of their position in food webs, top carnivores are important in the management and monitoring of whole ecological communities (Soulé & Kohm 1989; Gittleman & Pimm 1991).

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